UNIVERSITY
OF TASMANIA

STIM1 mediates multiple signalling pathways in neuronal growth cones

Camilla B Mitchell

BMedRes (Hons)

Submitted in fulfillment of the requirement for the
Degree of Doctor of Philosophy (Medical Research)

Menzies Research Institute Tasmania

University of Tasmania

February 2014

Declaration

This thesis contains no material which has been accepted for a degree or diploma by the University of Tasmania or any other institution, except where acknowledged, and by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

Figure 2.1 A was included in my honours thesis, Mitchell 2009, University of Tasmania

Figure 2.2 B was included in my honours thesis, Mitchell 2009, University of Tasmania

Camilla Mitchell

February 2014

Authority of Access

This thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968.

Camilla Mitchell

February 2014

Publications arising from this thesis

Mitchell CB, Gasperini RJ, Small DH, and Foa L. (2012) STIM1 is necessary for store-operated calcium entry in turning growth cones. *J Neurochem*, **122**, 1155-1166.

Statement regarding published work contained in this thesis

The publishers of the papers comprising chapters 2 (Figures 2.1, 2.2, 2.5 and 2.10) and chapters 3 (Figures 3.1, 3.2, 3.3, 3.4, 3.5 and 3.6) hold the copyright for that content, and access to the material should be sought from the respective journals. The remaining non-published content of the thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968.

Statement of Co-Authorship

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Name and School = **Camilla Bessie Mitchell,** Menzies Research Institute Tasmania
Name and institution = **Robert J Gasperini,** Menzies Research Institute Tasmania
Name and institution = **David H Small,** Menzies Research Institute Tasmania
Name and institution = **Lisa Foa,** School of Medicine and Menzies Research Institute Tasmania

Author details and their roles:

Mitchell CB, Gasperini RJ, Small DH and Foa L. (2012) ***STIM1 is necessary for store-operated calcium entry in turning growth cones,*** *J Neurochem,* ***122****, 1155-1166*

Data published in this paper is presented in this thesis in figures 2.1, 2.2, 2.5, 2.10, 3.1, 3.2, 3.3, 3.4, 3.5, and 3.6).

Percentage contribution of each author is outline in the table below:

Author	Research Performed	Data analysis	Paper writing	Designed project
CM	85	70	30	5
RJ	15	20	25	10
DS	0	5	5	5
LF	0	5	40	80

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Signed: _____ _____

Lisa Foa
Primary supervisor
Menzies Research Institute Tasmania
School Of Medicine
University of Tasmania

Head of School
Menzies Research Institute Tasmania
University of Tasmania

Date: 3/9/13

Statement of ethical conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

Camilla Mitchell

February 2014

Acknowledgements

Firstly, I'd like to express my sincere gratitude to my primary supervisor, Dr. Lisa Foa. Thank you for supporting me, teaching me and encouraging me when I was down. I am so grateful for your support, both intellectually and personally, and for the guidance you have given me over the past four and a half years. Thank you for believing in me, and only wanting the best for me. I'm extremely grateful that you never minded when I would be in your office every day, and sometimes multiple times a day. You got that demanding student you always wanted! And thanks for all of those Monday morning football debriefs.

To my co-supervisor, Dr Rob Gasperini, thank you for teaching me microscopy. Thank you so much for your help with my calcium imaging, and for taking over when I couldn't hack it anymore. Thank you for supporting me, helping me and encouraging me when I was having a tough time. I'm extremely grateful for your willingness to answer the late night calls when I was stuck on the scope and didn't know what I was doing.

To Professor David Small, thank you for your support and encouragement, and for being willing to help when needed. I'm grateful for the constructive criticism in lab meetings and for teaching me how to give a great presentation.

A big thanks to my PhD sister, Lila Landowski. Lils, you rock my world! How glad am I that we became the A-team, way back in second year physiology. Thank you for your

friendship, for your support, for the random lab gifts, and for all of the many, many, many chats, debriefs, and rants late in to the night in the turning room. You have kept me (mostly) sane, and I know I've got a forever friend!

To Edgar Dawkins, for your English entertainment and loads of laughs; Claire Hadrill, for the many hot chocolate adventures and always being my friend, even when you disagreed with my point of view; Adrian Thompson, thanks for being a great friend, my football buddy, and for coming back; Emily Ainslie, for being my lab niece, and my first (and best) vegan friend; Dr Dave Klaver, thanks for teaching me the ways of the lab, how to do a western, and always having a chat and a laugh.

To the rest of the Young Small Foians, both past and present, for all of your support, encouragement, and friendship, especially our lab mother Jenny Smith, for all you taught me.

To the honours group of 2009 who stuck around, especially Katherine Southam for being my write-up support and knowing exactly where I'm at (mentally!) all the time; to the Menzies crew, the crossword crew, and the tearoom crew, thanks for your friendship and all the fun times we had together.

Personally I would like to thank my parents for all of their love and support through my university life, for providing me with so much, so that I could just focus on my studies, and do the very best I could.

And finally, a big thank you to Cameron; for pulling me back into reality, keeping me sane, supporting me and encouraging me no matter how grumpy or upset I was. You really are my favourite. Thank you so much for loving me even though I've only been stressed since you met me. It can only get better from here!

Abstract

Calcium is an intracellular second messenger that is vital for normal neuronal function. The maintenance of calcium homeostasis is critical for healthy neuronal function, and disruption in calcium homeostasis has been implicated in diseases such as epilepsy and Alzheimer's disease. In developing neurons, calcium signalling regulates the precise wiring of neurons, in a process known as axon guidance. Axon guidance is extremely important in the normal healthy development of the nervous system. Aberrant axon guidance is highly associated with several neurodevelopmental disorders including autism and mental retardation syndromes such as fragile-X syndrome. Axons navigate the environment by a dynamic navigational structure located at the distal tip of an extending axon, known as a growth cone. Cytosolic calcium is crucial in mediating growth cone navigation. Correct understanding of the signalling mechanisms that regulate cytosolic calcium is key to understanding normal growth cone function.

This thesis focuses on the molecular mechanisms that regulate a vital source of calcium within growth cones, the endoplasmic reticulum (ER). Little is known about the function of the ER within growth cones. Stromal Interaction Molecule 1 (STIM1) is a calcium sensing protein in the ER membrane, which interacts with Orai proteins in the plasma membrane to initiate store-operated calcium entry (SOCE) and refill depleted intracellular calcium stores. The central hypothesis of this thesis is that STIM1 is necessary for SOCE in neuronal growth cones, and is required for axon guidance.

The results presented within this thesis demonstrate the presence and function of STIM1-mediated processes within the developing nervous system. This thesis has utilised primary cell culture of embryonic dorsal root ganglia neurons and immunocytochemistry to investigate the presence and localisation of STIM1 within developing growth cones. STIM1, along with its binding partners Orai1 and Orai2 reside in two different localisation patterns within growth cones; active (punctate) and inactive (diffuse). Depletion of calcium stores resulted in the activation of STIM1 within growth cones, increasing the number of growth cones displaying punctate STIM1 protein distribution. Calcium depletion also increased colocalisation between STIM1 and Orai1. Furthermore, STIM1 localisation appeared to be biased towards the turning side of the growth cone, in response to a calcium-dependent guidance cue. These data suggest that STIM1 and the Orai proteins are dynamic proteins that function in the regulation of calcium within growth cones.

While immunocytochemistry data suggested that STIM1 was functional within growth cones, a target morpholino approach was used to determine if STIM1 was necessary for growth cone function. A reduction of endogenous STIM1 reversed turning towards BDNF and netrin-1, and demonstrated that STIM1-mediated SOCE was necessary for BDNF signalling in growth cones. Unexpectedly, a reduction in STIM1 abolished turning away from Sema-3a in a manner independent of SOCE. In a growth cone collapse assay, STIM1 was also found to be necessary for Sema-3a-induced collapse, suggesting that STIM1 is implicated in multiple Sema-3a signalling pathways. This knockdown approach clearly demonstrates the necessity of STIM1 function for normal growth cone turning.

While the main function of STIM1 is thought to be the activation of Orai proteins, and subsequent activation of SOCE, STIM1 has been shown to interact with other signalling proteins, including the second messenger cAMP, in a process termed store-operated cAMP signalling. This study utilised cAMP analogues to determine if store-operated cAMP signalling was functional within growth cones. Upon the activation of cAMP, repulsive turning away from Sema-3a was restored in growth cones with reduced levels of STIM1. Sema-3a collapse was also prevented upon addition of cAMP agonists in control growth cones, but not restored in STIM1 morphants. Similar results were achieved with cGMP agonists. These data suggest that STIM1 mediates cyclic nucleotide signalling within growth cones. Furthermore, STIM1 has also recently been implicated in the reciprocal control of L-type voltage-gated calcium channels (VGCCs) and Orai proteins. While L-type VGCCs are important in mature neurons, there is conflicting data in the literature as to their role in axon guidance. This study investigated whether there was a potential interaction between STIM1 and L-type VGCCS in growth cones, and found that if there is an interaction, it is not essential for growth cone turning, but may be required for axon extension.

These results indicate a number of novel findings: Firstly, that STIM1 mediates growth cone navigation in response to both calcium-dependent and -independent guidance cues. Secondly, that STIM1 is required for Sema-3a signalling. Thirdly, that STIM1 mediates cyclic nucleotide signalling pathways within growth cones, and likely does not interact with

L-type VGCCs for growth cone navigation. In conclusion, this thesis has significantly added to the understanding of the regulation of the calcium signalling pathways that are crucial for normal growth cone guidance, enhancing our understanding of growth cone navigation, and in particular the regulation of the calcium signalling pathways that are crucial for normal growth cone guidance. These findings add to the pool of knowledge of how growth cones function and regulate calcium, which is crucial for normal neuronal health within development.

Table of Contents

Abbreviations List

$[Ca^{2+}]_i$	Intracellular calcium concentration
6-Phe-cAMPS	Sp-6-Phe-cAMPS
8-Me-cAMPS	8-pCPT-2'-O-Me-cAMPS
AC	Adenylate cyclase
Ach	Acetylcholine
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BDNF	Brain derived neurotrophic factor
CAD	CRAC activation domain
CaMKII	Calcium/calmodulin kinase II
cAMP	Cyclic adenosine monophosphate
CaN	Calcineurin
cGMP	Cyclic guanosine monophosphate
CIF	Calcium Influx Factor
CNS	Central nervous system
CRAC	Calcium-release activated calcium
CICR	Calcium-induced calcium release
DCC	Deleted in Colon Cancer
DRG	Dorsal root ganglia
EDTA	Ethylenediaminetetraacetic acid

EGTA	Ethylene glycol tetraacetic acid
Epac	Exchange protein directly activated by cAMP
ER	Endoplasmic reticulum
ER-PM	Endoplasmic reticulum-plasma membrane
ERM	ezrin/radixin/moesin
f-actin	Filamentous actin
FLIP	Focal laser-induced photolysis
GTP	Guanosine triphosphate
HBSS	Hank's Buffered Salt Solution
I_{CRAC}	CRAC current
IP_3	Inositol trisphosphate
IP_3R	IP3 receptor
IR	Immunoreactivity
MAG	Myelin-associated glycoprotein
MAPK	Mitogen-activated protein kinase
mRNA	messenger RNA
NaCl	Sodium Chloride
NFAT	Nuclear Factor of Activated T cells
NGF	Nerve Growth Factor
Npn	Neuropilin
NT-3	Neurotrophin 3

NT-4/5	Neurotrophin 4/5
P75ntr	p75 neurotrophin receptor
PBS	Phosphate buffered saline
PDE	Phosphodiesterase isozyme
PI3-K	Phosphoinositide 3-Kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein Kinase A
PKG	Protein Kinase G
PLC	Phospholipase C
PTEN	Phosphatase and tensin homolog
PVDF	polyvinylidene difluoride
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family, member A
RIPA	Radioimmunoprecipitation assay buffer
RNAi	RNA interference
ROI	Region of interest
RyR	Ryanodine Receptor
SAM	Sterile Alpha Motif
SCID	Severe Combined Immunodeficiency
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean

Sema-3a	Semaphorin-3a
SERCA	Sarco/endoplasmic reticulum
SNM	Sensory Neuron Media
SOC	Store- operated calcium
SOCC	Store- operated calcium channel
SOCE	Store-operated calcium entry
STIM	Stromal Interaction Molecule
Tg	Thapsigargin
TrK	Tyrosine Kinase
TRP	Transient receptor potential
TRPC	Transient receptor potential canonical
VGCC	Voltage-gated calcium channel
w/w	weight/weight
Xenopus	*Xenopus laevis*